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Quantitative studies of tubular immune complex formation and clearance in rats

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Quantitative studies of tubular immune complex formation and clearance in rats. Tubular antibody deposition and clearance was quantitatively studied using affinity-purified rabbit antibodies to rat Tamm-Horsfall protein (TH), a surface membrane glycoprotein of the tubular cells of the thick ascending limb of the loop of Henle. Immune complexes are formed in situ at the base of these cells in rats injected with antisera to TH. The renal binding of I^{125}-anti-TH was determined in pair label studies. Kidneys and other organs were removed from groups of rats for isotope counting at four hours to 14 days after an injection of I^{125}-anti-TH and I^{131}-normal rabbit IgG. The greatest total renal anti-TH binding after injection of 500 μg of anti-TH was observed at 24 hours in normal rats (18.55 ± 1.6 μg). During the period of most rapid clearance (day 2 to day 7) the half life of renal anti-TH binding (84.2 hours) and the half life of anti-TH in the serum (68.5 hours) were shorter than that of IgG in the serum (117.8 hours). There was no substantial uptake of anti-TH by other organs. A close relationship between serum levels and renal uptake of anti-TH at 24 hours was also observed in rats given from 50 to 6000 μg of anti-TH; renal saturation was evident only at the highest dose. This close relationship was also present during the clearance phase in rats injected with 3700 μg of anti-TH; the half life of anti-TH was 96.2 hours in kidneys and 110 hours in serum while the half life of rabbit IgG in serum was 151.8 hours. Markedly increased renal uptake of anti-TH was observed in proteinuric rats with passive Heymann nephritis. In very proteinuric rats, 14.1% of the injected dose was bound to kidneys at 24 hours. In these rats, serum anti-TH levels decreased very rapidly to 4% of control serum levels by five days. Throughout the period of study, the serum levels of anti-TH determined by direct radiometric assay corresponded very closely to those obtained by enzyme-linked immunosorbent assay (ELISA). Urinary excretion was a major mechanism for the clearance of anti-TH in proteinuric rats; more than 10% of the injected I^{125}-anti-TH was recovered intact (that is, protein bound) during the first day after injection. During the clearance phase for renal deposits, urinary clearance of anti-TH exceeded urinary clearance of IgG due to release of renal bound antibody into urine. These quantitative studies have defined a new pattern of renal antibody binding kinetics in normal rats which differs from patterns observed with intrinsic glomerular antigens; a close relationship between serum levels and renal binding of antibody over a broad dosage range was apparent during both uptake and clearance phases. They also show that TH is present on cell surfaces only in the kidney, and that increased glomerular permeability greatly alters the kinetics of tubular antibody deposition and clearance.

Immunopathologic studies in experimental animals and man have provided evidence that the pathogenesis of antibody-mediated tubulointerstitial nephropathies may involve antibodies to either basement membranes [1, 2] or tubular antigens [3–6]. Available evidence strongly indicates that the mechanism of immune deposit formation in these diseases primarily involves the in situ reaction of free circulating antibody with antigens already present within tubular basement membranes [1, 7] or along tubular cell surface membranes [8]. Tamm-Horsfall protein (TH) is a surface membrane glycoprotein of the cells of the thick ascending limb of the loop of Henle (TAL) [9, 10]. Subepithelial granular deposits of IgG, C3 and TH at the base of TAL cells are formed in rats passively immunized with heterologous antisera to TH [6]; in the presence of proteinuria, immune deposits are also formed on the luminal surfaces of TAL cells [11].

Quantitative analysis of renal subepithelial immune deposit formation in glomerular models such as serum sickness in rabbits [12, 13] and passive Heymann nephritis in rats [14, 15], as well as other glomerular models involving endogenous [16–18] and planted exogenous antigens [19, 20] has been previously reported, but a comparable analysis of tubular subepithelial immune complex formation has not been previously described. Since a single injection of antibody to TH induces tubular immune deposits in rats, we were afforded the opportunity to quantitatively measure the kinetics of net tubular immune deposit formation and clearance using pair-label radioisotope techniques. We were also able to quantitatively evaluate the influence of altered glomerular permeability on these kinetics. The present studies quantitatively define a unique time course of subepithelial renal immune deposit formation and clearance.

Methods

Male Sprague-Dawley rats initially weighing approximately 100 grams were used in these studies. Rats were anesthetized with a mixture of ketamine and xylazine [21] prior to obtaining blood and tissue samples. Rat TH was isolated from normal rat urine and rabbit antisera to rat TH (anti-TH), euglobulin and IgG fractions of anti-TH were prepared as previously described [6, 22]. Sheep antisera to rat proximal tubular fraction 1A (anti-Fx1A) was prepared as previously described [23]. Serum levels and urinary excretion of rat albumin and rabbit IgG were determined by single radial immunodiffusion [24]. Specimens for the quantitative determination of urinary protein and antibody excretion were obtained in metabolic cages with sodium

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azide (final concentration of .02%) in the receiving containers. Renal tissues were processed for and evaluated by immunofluorescence microscopy as previously described [5].

Purification of anti-TH antibodies by affinity chromatography

Solid phase immunoabsorbents were prepared by conjugating rat TH to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey, USA). In order to avoid the aggregation of TH under the conditions used for conjugation, rat TH was irreversibly disaggregated with 50% acetic acid [25] prior to conjugation, dialyzed against deionized water and lyophilized. A single line was observed after immunodiffusion in 1% agarose of this protein in 0.01 M phosphate 0.15 M NaCl pH 7.0 (PBS) against anti-TH. Rat TH (8.5 mg) prepared in this way was coupled to 5 ml of Sepharose 4B activated with cyanogen bromide as described by March, Parikh and Cuatrecasas [26]. This TH-sepharose gel was extensively washed with PBS and further washed with high and low pH buffers and inactivated with glycine as described [26]. Approximately 40% of the protein used for coupling was recovered in these buffer washes of the gel. For immunoabsorbent purification, 10 ml of rabbit antisera to rat TH was mixed with 5 ml of TH-sepharose gel, and incubated overnight with shaking at 4°C. The gel was washed with PBS and then eluted using a batch method with a 0.1 M glycine buffer pH 2.8 for 30 minutes. The eluate (22 ml) was immediately neutralized with a 1 M $NaHCO_3$ buffer pH 8.0 and then dialyzed against PBS. The rabbit IgG content of affinity-purified antibody solutions was determined by single radial immunodiffusion and confirmed by the Lowry method. Anti-TH antibodies were concentrated under reduced pressure to 2 mg/ml using Sartorius membrane filters (Brinkman Instruments, Inc., Westbury, New York, USA) and stored in aliquots at −20°C.

Radiolabelling of anti-TH antibodies and normal rabbit IgG

Anti-TH, purified as described above, was labelled with I^{125} using the chloramine T method as modified by McConahey and Dixon [27]. Anti-TH (500 μg of IgG) in 0.25 ml of 0.1 M phosphate buffer, pH 7.4, and 500 μCi of I^{125} (Amersham, Arlington Heights, Illinois, USA) (25 μl) were reacted with chloramine T (100 μg) for 10 minutes in a glass tube within an ice bath. The reaction was stopped by adding 100 μg of sodium metabisulfate in 200 μl. The solution was passed through Sephadex G-25 (Pharmacia) and dialyzed against PBS to further remove free iodine.

The I^{125} in the radiolabelled anti-TH preparation used in these studies was shown by TCA precipitation to be 99% protein bound. The specific activities of iodinated antibody preparations were 1.1 to 1.3 × 10^6 CPM/μg of protein. Normal rabbit IgG was iodinated with I^{131} by the same method. The I^{131} content of these rabbit IgG preparations was 98% TCA precipitable. After determining specific activity, a 20-fold excess of normal rabbit IgG was added to each of the iodinated protein solutions to minimize denaturation. Prior to injection, I^{131}-normal IgG was mixed with I^{125}-anti-TH and cold anti-TH in ratios giving roughly equivalent cpms for I^{125} and I^{131} and a total of 50 to 6000 μg of anti-TH/ml.

Influence of iodination on anti-TH antibody activity

The antibody activity of radiolabelled anti-TH was evaluated by immunoabsorption studies using TH-sepharose. In polystyrene test tubes, 0.1 ml of gel was mixed with 10 μl of I^{125}-anti-TH and counted in duplicate in a Beckman 7000 automated gamma-counter (Beckman Instruments, Inc., Palo Alto, California, USA). After two hours of incubation, the gel was washed twice with 2 ml of PBS and centrifuged for five minutes at 1,000 rpm. After the second wash, the gel was quantitatively transferred to a new tube. A total of 76 to 80% of counts were bound to the TH-sepharose gels, while less than 3% of total counts were present in the second washes. The recovery of initial counts from the combination of gels, the original tubes and the two supernatants was 98%. Control studies using rat euglobulins coupled to Sepharose or uncoupled Sepharose were performed at the same time. Only 1.7% of counts were bound to the euglobulin gels and 1.3% of counts were bound to uncoupled Sepharose; 94% of initial counts were recovered with both of these gels. The binding of I^{131}-normal rabbit IgG to Sepharose and to Sepharose-staphylococcal protein A (Pharmacia) was examined in a similar way. Only 1% was bound to Sepharose, while an average of 77% of I^{131}-normal rabbit IgG was bound to the protein A gels. Recovery of original counts was more than 96% in each of these studies.

Tissue uptake of anti-TH antibodies

Potassium iodide, 5 mg/100 ml and sodium chloride, 0.45 g/100 ml were added to the drinking water of rats starting five days prior to the injection of radiolabelled anti-TH and continued until sacrifice. Under anesthesia, both kidneys and the liver and spleen were removed, blotted and weighed after blood had been obtained by cardiac puncture. In selected animals, the lungs, thymus, thyroid, heart, brain and bladder were also removed for counting. Changing the orientation of tissues in the scintillation vials within the automated gamma-counter or homogenization of kidneys by dicing prior to counting a second time did not alter the numbers of counts observed in a total of 14 initial individual samples studied. Therefore, subsequent studies were performed on non-homogenized organs. Specific renal binding of anti-TH antibodies was calculated by the following equation for pair label studies [19]:

$$\text{Kidney bound anti-TH} = \text{Kidney } I^{125} - \frac{\text{Serum } I^{125} \times \text{Kidney } I^{131}}{\text{Serum } I^{131}}$$

More than 98% of the radioactive counts in serum were precipitated with 20% TCA and counts were adjusted for decay based on aliquots of the iodinated proteins used as standards and counted initially and on each day that additional serum or tissue samples were counted. The binding of anti-TH antibodies in other organs was calculated using the above equation for kidney binding. Results are expressed as mean ± SEM. Half lives were calculated from the slopes of the exponential curves determined using scatter plots.

Urinary excretion of anti-TH and IgG

Duplicate 1 ml aliquots of each 24-hour urine collection were transferred to polystyrene tubes and counted to determine total

urinary I^{125} excretion per ml. After mixing with 150 μl of normal rat serum to provide a large protein pellet, 1 ml of 20% TCA was added to each tube. Tubes were centrifuged for five minutes at 3000 rpm and the supernatant was removed and counted. An additional 3 ml of 20% TCA was added to the precipitate. After mixing and recentrifugation, the supernatant and final precipitate were separated and counted for I^{125}. The urinary volume was multiplied by the number of I^{125} cpm in the final precipitate to determine the urinary excretion of protein-bound I^{125} and by the combined counts in the supernatants after TCA precipitation to determine unbound I^{125} excretion. The combined recovery of counts in the supernatants and precipitate was consistently 92 to 98% of total urinary I^{125} measured prior to precipitation.

Total urinary excretion of protein bound I^{131} and unbound I^{131} were similarly determined and results expressed as mean ± 1 SD. Urinary clearances were calculated using serum levels for the midpoints of clearance periods determined from exponential plots.

Enzyme-linked immunosorbent assay (ELISA) of anti-TH in serum

Immunoassays for the determination of serum levels of rabbit antibodies to rat TH were performed using the ELISA method previously described [28] using polystyrene tubes coated with rat TH, alkaline phosphatase-conjugated goat anti-rabbit IgG as the detecting antibody, and affinity purified anti-TH as standards. This method for determining serum levels was not satisfactory for quantitatively measuring anti-TH in urine since urinary TH competitively inhibited the binding of anti-TH to antigen-coated tubes.

Experimental design

Uptake and clearance of anti-TH in normal rats

Thirty-nine normal rats received of 6 mg of rabbit IgG in PBS that contained a total of 500 μg of trace labelled I^{125}-IgG antibodies to rat TH (approximately 2×10^6 cpm/rat of I^{125}) and contained an equivalent quantity of I^{131}-normal rabbit IgG as a pair label. These rats were bled for baseline serum isotope counts at one hour after injection and sacrificed in groups of two to nine rats at 4, 8, and 16 hours, and at 1, 3, 5, 7, and 14 days after injection. Whole kidneys and other organs were removed, weighed wet and counted along with standards and serum obtained at each interval after injection.

Influence of dose of anti-TH on uptake and clearance in normal rats

Fifty-four normal rats received an intravenous injection of one ml of trace labelled I^{125}-anti-rat TH and trace labelled I^{131}-normal rabbit IgG containing a total from 50 to 6000 μg of carrier anti-TH in the form of antisera or a euglobulin fraction of antisera containing 10 mg/ml and 20 mg/ml of rabbit IgG, respectively. In the groups of rats that received less than one ml of antisera (8 groups of 4 to 5 rats) or euglobulin fraction of antisera (4 groups of 3 to 5 rats), the volume was brought to one ml by the addition of normal rabbit serum or the euglobulin fraction of normal rabbit serum, respectively. These rats were sacrificed 24 hours later to obtain renal tissue and sera for isotope counting. Six groups containing 25 rats received less than the standard dose of 500 μg of carrier anti-TH and six groups containing 29 rats received from 600 to 6000 μg of carrier anti-TH.

The effect of an increased dose of anti-TH on the pattern of uptake and clearance of anti-TH was studied in 22 additional rats injected with one ml of trace labelled I^{125}-anti-rat TH and trace labelled I^{131}-normal rabbit IgG and a euglobulin fraction containing 3700 μg of carrier anti-TH and 30 mg/ml of rabbit IgG. Groups of four rats each at 1, 2, 3, and 5 days and groups of three rats each at 7 and 12 days were sacrificed to obtain renal tissue and sera for isotope counting.

Influence of proteinuria on uptake and clearance of anti-TH

Twenty-one rats were injected intravenously with sheep antisera to Fx1A to produce heterologous immune complex nephritis (HICN). Ten days later, these proteinuric rats and nine litter mates received either 6 mg (5 control and 10 HICN rats) or 10.8 mg (4 control and 11 HICN rats) of rabbit IgG containing I^{131}-normal rabbit IgG and 500 μg of I^{125}-anti-TH. Two quantities of carrier IgG were used to allow direct comparison of studies with those in normal rats described above and with our previous morphologic studies of HICN rats given anti-TH [11] in which the larger quantity of carrier IgG was used. Groups of rats were bled for baseline counts at one hour and at the time of sacrifice for tissue binding studies at four hours, one day, three days, and five days after injection. Urinary excretion of protein bound and unbound I^{125} and I^{131} was determined on the daily samples obtained throughout the experiments to provide data concerning the metabolic fate of radiolabelled proteins.

On the tenth day after 27 additional proteinuric HICN rats had received anti-Fx1A, these rats and 17 non-proteinuric control rats were also injected with 1 ml of antisera containing 500 μg of rabbit anti-TH. Renal histology was evaluated at comparable intervals and serum levels of anti-TH were determined by ELISA.

Results

General observations

All HICN rats were proteinuric by the tenth day when anti-TH was injected. Since there was a considerable range of proteinuria (from 33 to 260 mg/day in the radiometric experiments and from 48 to 334 mg/day in the ELISA experiments), these rats were divided into two groups for the analysis. Albuminuria in the group of more highly proteinuric rats in each set of experiments was greater than 150 mg/day during the time from one day prior to anti-TH until sacrifice. Albuminuria in this group was 209 ± 31.3 mg/day in the radiometric experiments and 277 ± 65.5 mg/day in the experiments analyzed by ELISA. The corresponding groups of HICN rats with less albuminuria had 106 ± 34.6 mg/day and 109 ± 23.5 mg/day, respectively. All control rats excreted less than 1.0 mg of albumin per day.

Immunofluorescence microscopy

The localization and time course of formation and clearance of granular immune deposits of rabbit IgG at the base of thick ascending limb of the loop of Henle (TAL) cells (Fig. 1) in kidneys of normal rats injected with anti-TH was identical to

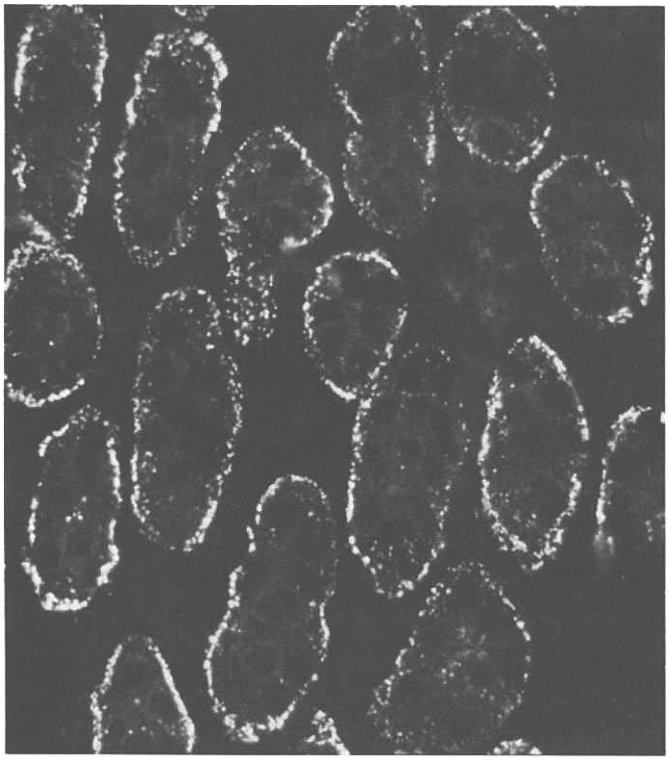

Fig. 1. *Granular IgG deposits at the base of tubular cells of the thick ascending limb of the loop of Henle in kidney of normal rat at one day after injection with anti-TH.* (anti-rabbit IgG, × 400).

Fig. 2. *Tubular immune deposits in kidney of proteinuric HICN rat at one day after injection with rabbit anti-TH.* Prominent linear IgG deposits are present on luminal surfaces of the cells of the thick ascending limb of the loop of Henle while granular deposits are present at the base of these cells. (anti-rabbit IgG, × 420).

those previously described [6]. The IgG deposits were maximal during the first few days after injection and had been nearly completely cleared by 14 days. The luminal tubular deposits (Fig. 2) in HICN rats injected with anti-TH were also identical in localization to those previously described [11]. The large luminal IgG deposits were maximal at one day after injection in the more proteinuric HICN rats, while these deposits were maximal at three days in the less proteinuric group of HICN rats. These luminal deposits were also nearly completely cleared in both groups by the end of two weeks. The granular glomerular capillary wall immune deposits of immunoglobulins in HICN rats were identical to previously described findings in this model [11, 23].

Organ uptake and clearance of anti-TH-I^{125}

The time course of renal uptake and clearance of I^{125}-anti-TH in normal rats is shown in Figure 3. The phase of net anti-TH uptake occurred during the first day after injection. More than 58% of peak anti-TH binding was observed at eight hours (10.80 ± 1.93 μg). Maximal binding (18.55 ± 1.60 μg) was observed in the nine rats studied at 24 hours after injection. By five days, the renal binding of anti-TH had decreased to nearly half of peak levels (9.1 ± 0.14 μg). This decline of anti-TH binding continued over the study to 1.65 ± 0.3 μg at 14 days. The pattern of renal uptake and clearance of anti-TH was similar when results were expressed as anti-TH bound per gram of kidney rather than per total renal mass with a mean peak of

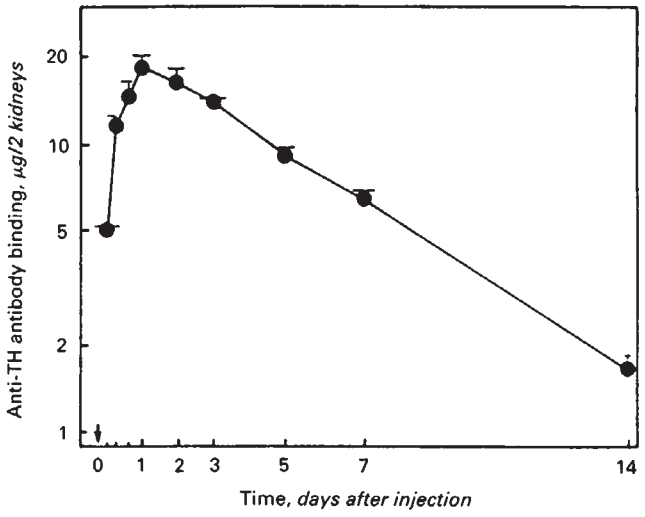

Fig. 3. *Renal binding of I^{125}-anti-TH (mean ± SEM) in normal rats injected intravenously with 500 μg of anti-TH.* Each point represents 4 to 9 rats studied except at 4 hours (3 rats) and 7 and 14 days (2 rats each).

13.88 μg of anti-TH/gram observed at 24 hours. At all times from 8 hours to day 14, the mean binding of I^{125}-anti-TH to the livers and spleens of these same groups of normal rats showed no substantial net uptake (<0.5 μg/organ). The mean tissue

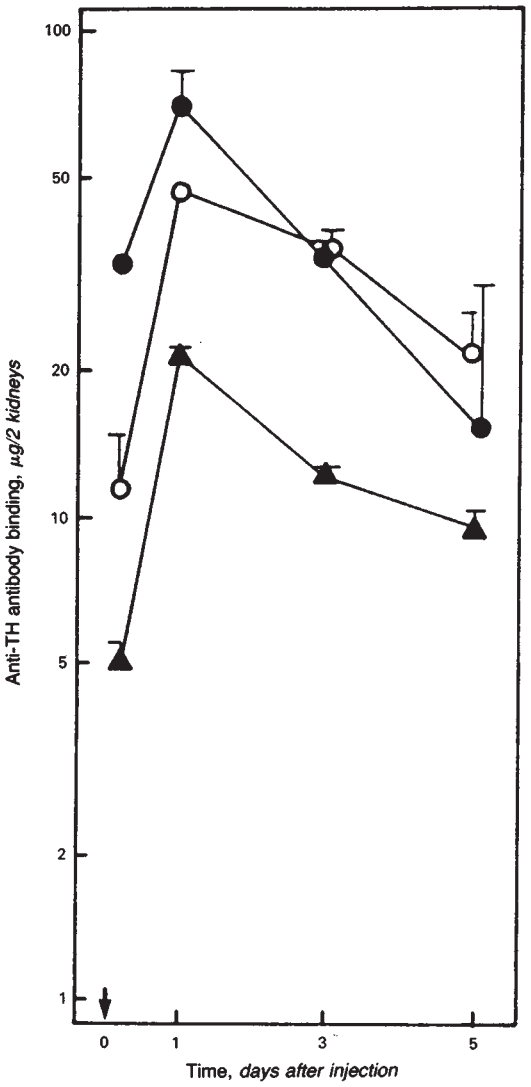

Fig. 4. *Renal binding of I^{125}-anti-TH (mean ± SEM) in nonproteinuric control rats (triangles) and heavily proteinuric (>150 mg/day) HICN rats (closed circles) and less proteinuric HICN rats (open circles) injected intravenously with 500 μg of anti-TH.* All points represent 2 to 4 rats except the open circle at 4 hours and the closed circle at 1 day for single rats.

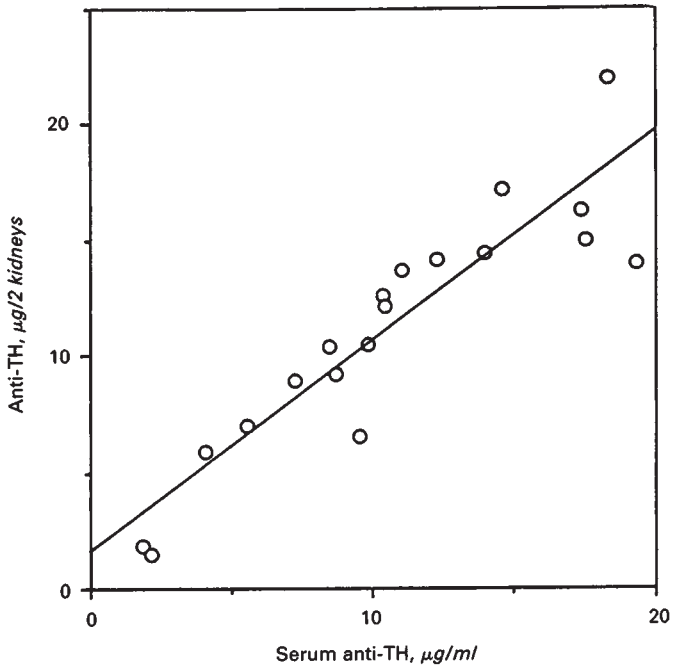

Fig. 5. *Serum levels and renal binding of I^{125}-anti-TH of individual normal rats during the clearance phase from day 2 to day 14 in the rats shown in Figure 3.* (y = 1.584 + 0.902x, r = 0.91)

uptake in the lungs and bladder of groups of four rats studied at 1, 2 and 3 days after injection was always less than 0.2 μg/organ; mean uptake in groups of two rats was less than 0.1 μg I^{125}-anti-TH/organ in the thymus, heart and brain at 1 and 2 days and the thyroid on day 3. At all times from four hours when mean kidney binding was 5.1 μg, and 7 days the net content of antibody in the liver and spleen was less than 3% of kidney binding. The total liver content at four hours was 0.23% of the injected dose (0.04%/g of liver); otherwise, the binding by liver or spleen was always less than 0.1% of the injected dose of antibody.

Increasing the injected dose resulted in proportionately greater renal uptake of anti-TH/2 kidneys at 24 hours over the entire dose range studied ($y = 0.4538 + 0.0316x$, $r = 0.958$). The I^{125}-anti-TH binding of the five rats given the highest dose was 185.7 ± 10.7 μg/2 kidneys. Renal clearance of anti-TH in the high dose clearance study was slightly less rapid; renal binding at five days was 58% of the peak at 24 hours versus 49% with the standard dose.

The time course of renal uptake and clearance of I^{125}-anti-TH in groups of proteinuric HICN rats and nonproteinuric control rats is shown in Figure 4. The binding of anti-TH in both groups of HICN rats greatly exceeded that in control rats, reflecting the anti-TH bound at both luminal and basal tubular surfaces in HICN rats (Fig. 2) while only basal anti-TH deposits are present in nonproteinuric rats (Fig. 1). At 24 hours, a peak value of 70.5 ± 12.5 μg of anti-TH was bound in the kidneys of the group of highly proteinuric HICN rats (14.1% of injected dose). The renal antibody binding in the less proteinuric group of HICN rats was more than twice that of controls at all times and at 5 days (21.1 ± 1.1 μg) exceeded the binding of the highly proteinuric group (15.1 ± 3.0 μg). The uptake of anti-TH by liver and spleen was not substantially altered in HICN rats. The mean liver uptakes in these groups of proteinuric rats studied at four hours and 1, 3 and 5 days after injection of I^{125}-anti-TH was always less than 1.0 μg (<0.2% of injected dose) and the mean spleen uptake in these rats did not exceed 0.2 μg at any time.

Serum levels

A comparison of serum levels (μg/ml) to renal binding of I^{125}-anti-TH in individual rats during the clearance phase from days 2 to 14 after injection of 500 μg of anti-TH in the long-term study of normal rats is shown in Figure 5. The half life of renal anti-TH binding during the most rapid clearance phase (from day 2 to day 7) was 84.2 hours (r = 0.83). During this period, the clearance of anti-TH from the serum (half life of 68.5 hours, r =

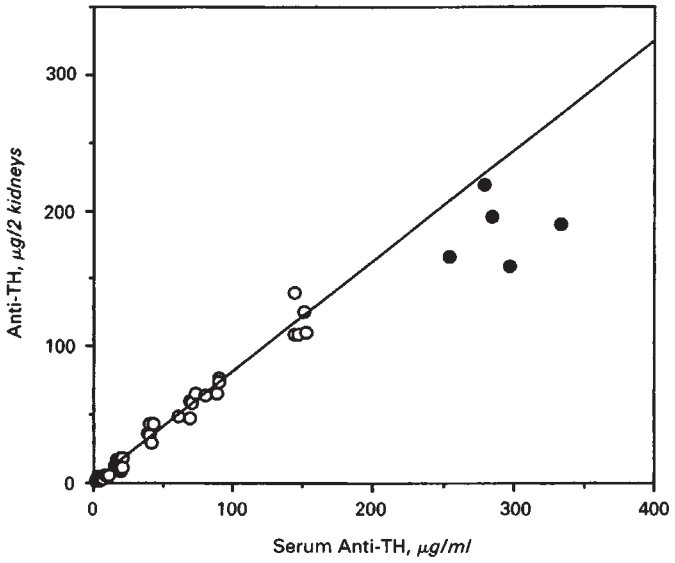

Fig. 6. *Serum levels and renal binding of I^{125}-anti-TH in 54 normal rats at 24 hours after intravenous injection of 50 to 6000 μg of anti-TH.* All rats in the highest dose group (closed circles) fall below the regression line for the rats given up to 3000 μg (open circles) ($y = -0.307 \pm 0.810x$, $r = 0.98$).

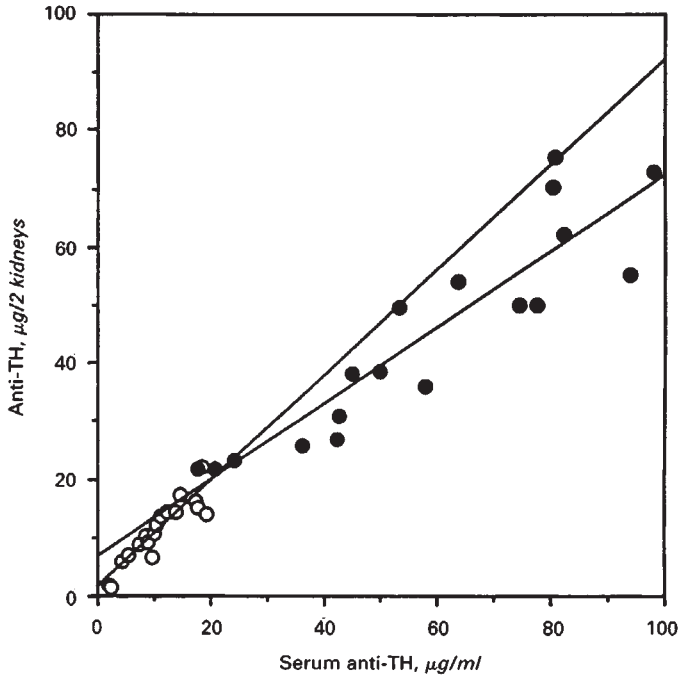

Fig. 7. *Serum levels and renal binding of I^{125}-anti-TH during the clearance phase from day 2 to day 12 in normal rats injected with 3700 μg of anti-TH (closed circles) ($y = 6.488 \pm 0.658x$, $r = 0.84$) and the rats injected with 500 μg of anti-TH (open circles) that are shown in Figure 5.*

0.89) was more rapid than clearance of I^{131}-IgG from the serum (half life of 117.8 hours, r = 0.77).

The relationship of serum levels to renal binding that was apparent during the clearance phase was further examined in additional groups. A comparison of serum levels and renal binding of I^{125}-anti-TH at the time of peak uptake at 24 hours in 12 groups of three to five rats that received 50 to 6000 μg of anti-TH is shown in Figure 6. Saturation of renal capacity for antibody binding was apparent only at the highest dose. All of the 25 rats given less than 500 μg of anti-TH had serum anti-TH levels that were less than 25 μg/ml. The slope of the regression line for this subgroup ($y = -0.534 + 0.767x$, $r = 0.86$) is very close to that of the entire group shown in Figure 6. A plot of serum levels and renal binding of I^{125}-anti-TH of rats during the clearance phase from day 2 to 12 after injection of a much larger dose of anti-TH (3700 μg) is shown in Figure 7. For comparison, the values for rats given the standard dose are also shown in this Figure. The half life of I^{125}-anti-TH in kidneys after the larger dose during the most rapid period of clearance from two to seven days was 96.2 hours (r = 0.90), while the half life of anti-TH in serum was 110 hours (r = 0.91) and the half life of rabbit IgG in serum was 151.8 hours (r = 0.87).

In the short-term study of HICN rats, the serum levels of I^{125}-anti-TH achieved at one hour after injection were similar in all groups: 44.9 ± 11.9 μg/ml (mean ± 1 SD) in six nonproteinuric control rats, 48.1 ± 10.4 μg/ml in 10 highly proteinuric (>150 mg/day) HICN rats, and 46.1 ± 8.1 μg/ml in eight less proteinuric HICN rats. By one day after injection, the mean serum level in highly proteinuric HICN rats (7.2 μg/ml) had decreased to half of that in control rats (14.2 μg/ml) and continued to rapidly decline (Fig. 8). By five days, the mean level of I^{125}-anti-TH (0.34 μg/ml) was 4% of the mean level in nonproteinuric control rats (8.0 μg/ml).

The decrease in serum levels of anti-TH measured by ELISA (Fig. 9) over time in each of the groups of rats was very similar to that observed in the radiometric study. Initial levels by ELISA at four hours of 30.3 ± 2.6 μg/ml (mean ± SEM) in control rats and 32.4 ± 1.8 μg/ml in highly proteinuric HICN rats were comparable. By one day after injection, the mean level in highly proteinuric HICN rats (5.15 μg/ml) was only one-third of the level in control rats (15.6 μg/ml), and by 7 days was less than 3% of the level of 4.5 μg/ml in nonproteinuric control rats. The half lives of anti-TH in the serum in the combined group of proteinuric rats by ELISA were comparable to those observed by radiometric assay (Table 1). The half life of anti-TH in serum in each group was shorter than that of IgG during each time period.

Serum albumin levels in all groups remained relatively constant over time. The mean level in the I^{125}-anti-TH group of

Table 1. Half lives in HICN rats

Days	I^{125}-rabbit-anti-TH		I^{131}-Normal rabbit IgG	Serum ELISA	
	Kidney[a]	Serum	Serum	Anti-TH	IgG
1–3	55.7	27.2	31.9	13.3	20.1
3–5[b]	24.6	51.2	75.2		
3–7[c]				56.3	141
1–5[b]	32.9	35.7	45.1		
1–7[c]				28.1	49.1

[a] half lives for anti-TH/2 kidneys
[b] Radiometric half lifes for the periods to day 5
[c] ELISA half lives are for the periods to day 3 or to day 7

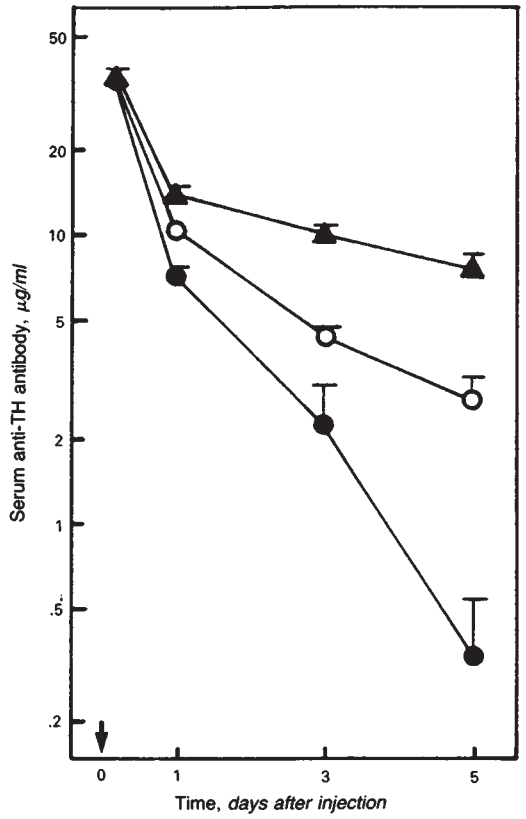

Fig. 8. *Serum levels of I^{125}-anti-TH (mean ± SEM) in the groups of nonproteinuric control rats (triangles), highly proteinuric (>150 mg/day) HICN rats (closed circles) and less proteinuric HICN rats (open circles) that are shown in Figure 4.*

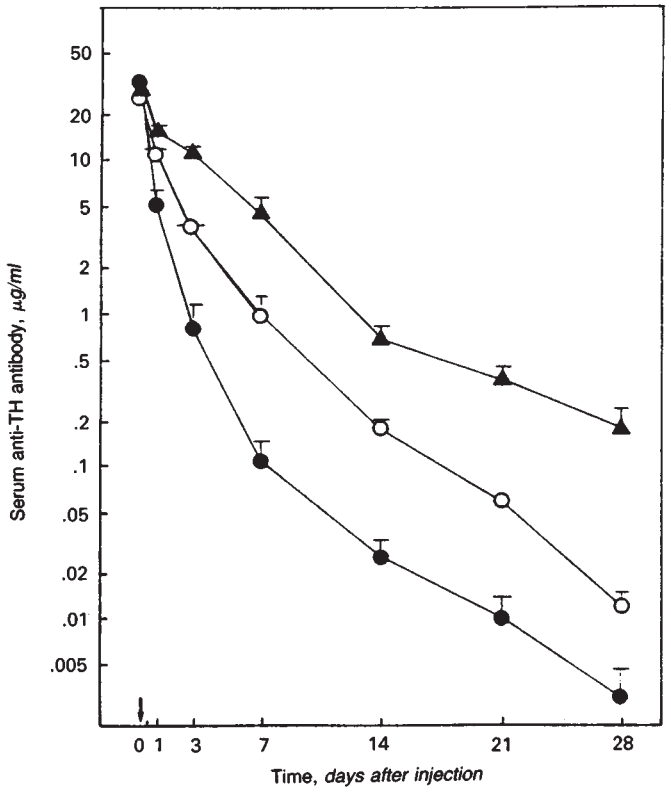

Fig. 9. *Serum levels of anti-TH (mean ± SEM) determined by ELISA in a total of 45 samples from highly proteinuric (>150 mg/day) HICN rats (closed circles), 19 samples from less proteinuric HICN rats (open circles) and 34 samples from nonproteinuric control rats (triangles).* All points in the control and highly proteinuric HICN groups represent 4 to 9 rats except at 28 days when 2 control and 3 HICN rats were studied. All points in the less proteinuric HICN group except 21 days reflect 2 or more rats.

highly proteinuric HICN rats of 11.1 ± 4.0 mg/ml was approximately half that of control rats (25.3 ± 3.3 mg/ml).

Urinary excretion of iodinated proteins

The urinary excretion of anti-TH during the five days after injection of 500 μg of I^{125}-anti-TH is shown in Table 2. The mean cumulative antibody excretion during the first three days was 79.2 μg in the more proteinuric group of HICN rats. The less proteinuric HICN group excreted approximately half as much I^{125}-anti-TH during this time and the nonproteinuric controls excreted a total of 2.8 μg (less than 0.6% of the injected dose). Substantial quantities of unbound I^{125} appeared in the urine of all groups. While less than 2% of the total urinary I^{125} excreted by controls during the five days of study was protein bound, more than 36% of isotope excreted by the more proteinuric HICN group and 19% by the less proteinuric HICN group was protein bound.

While 70% of urinary I^{131} excreted over the first three days by the more proteinuric HICN rats was protein bound, only 1% of I^{131} excreted by control rats during this period was protein bound (Table 3). These control rats excreted a total of 16% of injected I^{131} over the first three days, while the less and more proteinuric HICN groups excreted 47% and 62%, respectively, of the injected dose over this period. In control rats, 24% of the injected I^{131} was recovered in the urine as unbound isotope while 31% and 21%, respectively, in the less and more proteinuric groups of HICN rats was recovered in the urine as unbound isotope during the five days of study.

The ratio of I^{125}-anti-TH clearance to I^{131}-IgG clearance into the urine increased over the course of the study in both groups of HICN rats. In the more proteinuric group, the clearance of anti-TH rose to nearly twice that of IgG (Fig. 10).

The cumulative recovery of injected isotope based on the summation of the urinary excretion over five days plus the quantities measured in kidneys and other organs, plus the serum level multiplied by an estimated distribution factor of 0.15 × body weight [29] in six normal control rats was 78.9 ± 7.4% (mean ± 1 SD) for I^{125} and 99.3 ± 10.2% for I^{131}. In seven rats of the low proteinuric HICN group, recovery of I^{125} was 66.8 ± 7.1% and of I^{131} was 76.9 ± 8.0%, while in 10 rats of the highly proteinuric HICN group, recovery was 63.8 ± 9.9% for I^{125} and 75.3 ± 9.7% for I^{131}.

Discussion

The first quantitative analysis of subepithelial renal tubular immune complex formation is provided by the present studies. These studies have defined a unique relationship between immune deposits and serum antibody levels to an intrinsic renal antigen. Our previous immunofluorescence studies of the active and passive anti-TH models [5, 6, 30] had suggested that the

Table 2. Urinary excretion of I^{125} ($\mu g/day$)

Day		Control rats	HICN rats: Low proteinuria	HICN rats: High proteinuria
1	I^{125}-aTH	1.5 ± 0.3	21.5 ± 16.4	54.9 ± 24.4
	free I^{125}	55.2 ± 3.4	74.2 ± 24.0	92.4 ± 13.2
		(2)	(3)	(6)
2	I^{125}-aTH	0.8 ± 0.2	9.6 ± 1.7	18.3 ± 6.3
	free I^{125}	42.2 ± 0.8	65.7 ± 26.8	37.6 ± 13.5
		(2)	(3)	(4)
3	I^{125}-aTH	0.5 ± 0.1	6.7 ± 2.0	6.0 ± 2.3
	free I^{125}	38.3 ± 6.2	31.9 ± 7.7	14.1 ± 6.5
		(2)	(3)	(4)
4	I^{125}-aTH	0	8.7 ± 4.7	2.8 ± 1.3
	free I^{125}	21.6 ± 9.0	21.0 ± 10.4	8.1 ± 6.0
		(2)	(2)	(2)
5	I^{125}-aTH	0	2.5 ± 1.2	2.2 ± 1.4
	free I^{125}	23.0 ± 2.6	15.2 ± 8.4	3.9 ± 3.0
		(2)	(2)	(2)
Mean total excreted in 5 days	I^{125}-aTH	2.8	49	84.2
	free I^{125}	180.3	208	156.1

All rats were injected with 500 μg of I^{125}-anti-TH. Values are expressed as mean ± 1 SD. The number of rats in each group is given in parentheses.

Table 3. Urinary excretion of I^{131} ($\mu g/day$)

Day		Control rats	HICN rats: Low proteinuria	HICN rats: High proteinuria
1	I^{131}-IgG	15 ± 5	1530 ± 1138	3810 ± 727
	free I^{131}	620 ± 55	1133 ± 406	1300 ± 307
		(2)	(3)	(6)
2	I^{131}-IgG	5 ± 2	600 ± 78	740 ± 117
	free I^{131}	530 ± 60	980 ± 380	510 ± 270
		(2)	(3)	(4)
3	I^{131}-IgG	0	290 ± 26	180 ± 138
	free I^{131}	520 ± 160	570 ± 180	210 ± 144
		(2)	(3)	(4)
4	I^{131}-IgG	0	220 ± 130	150 ± 150
	free I^{131}	510 ± 85	410 ± 230	140 ± 140
		(2)	(2)	(2)
5	I^{131}-IgG	0	210 ± 150	50 ± 45
	free I^{131}	450 ± 125	290 ± 240	70 ± 70
		(2)	(2)	(2)
Mean total excreted in 5 days	I^{131}-IgG	20	2850	4930
	free I^{131}	2630	3383	2230

All rats were injected with 10,800 μg of I^{131}-rabbit IgG. Values are expressed as mean ± 1 SD. The number of rats in each group is given in parentheses.

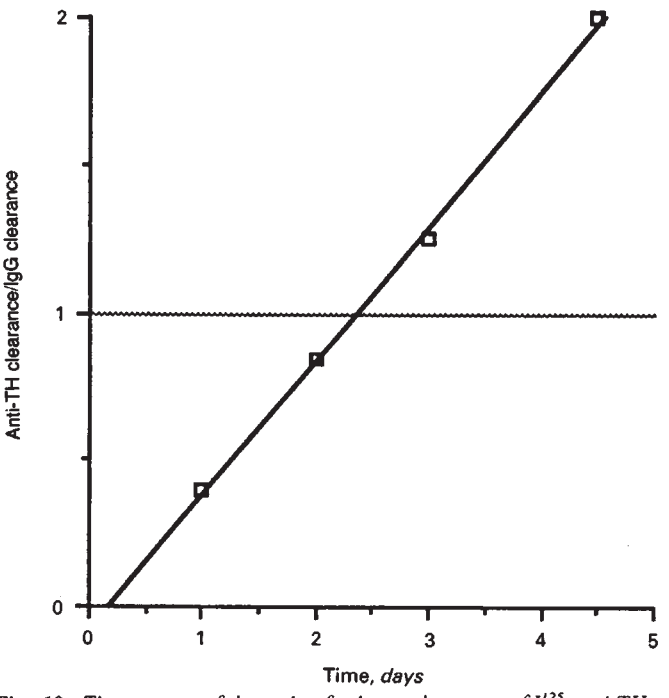

Fig. 10. *Time course of the ratio of urinary clearance of I^{125}-anti-TH to that of I^{131}-rabbit IgG in highly proteinuric HICN rats ($y = -0.081 + 0.457x$, $r = 1.00$).* Since only 2 rats were studied on days 4 and 5, results from these days were combined. Values greater than 1.0 reflect selective clearance of anti-TH into urine after day 2.

quantity of renal immune deposits and serum levels of anti-TH were closely related. Sequential studies of the tissue binding of I^{125}-anti-TH allowed us to more precisely demonstrate a close correspondence of serum antibody levels and renal binding over an extended period of time after injection of 500 μg (Fig. 5) and 3700 μg of anti-TH (Fig. 7). These studies also provided evidence that selective removal from the serum and renal uptake of anti-TH was continuing during the clearance phase; the half life of anti-TH in the serum was substantially shorter than that of rabbit IgG during the period of net clearance in both studies. The dose response study showed a close relationship between serum levels and renal uptake of anti-TH (Fig. 6). Evidence for renal saturation was apparent only at the highest dose. This partial dissociation of renal binding from serum levels resulted from disproportionately high serum levels at this dose rather than from lower renal uptake relative to dose. These kinetics of tubular immune complex formation and clearance differ substantially from those previously demonstrated for other intrinsic renal antigens in models of glomerular disease [14, 16]. The time course of net renal anti-TH antibody uptake to a peak at 24 hours is intermediate to the very rapid uptake phase, lasting less than an hour in the anti-GBM model [16] and net uptake of antibodies continuing for at least five days in the passive Heymann model [14]. However, antibody clearance in the passive TH model is remarkably more rapid than in either of these models of glomerular disease [16, 30–32].

Several aspects of the methodology employed in the present studies were advantageous and deserve comment. These include the use of anti-TH antibodies that had been affinity purified using TH-Sepharose gels and validation by the comparison of results obtained by direct radiometric assay with those obtained by ELISA. Affinity purification of anti-TH antibodies prior to radiolabelling allowed the percentage of injected antibodies that were bound to renal tissue to be precisely defined and permitted a more than 10-fold reduction of non-specific I^{125} counts injected. Essentially complete retention of antibody activity after iodination was demonstrated by in vitro absorption studies using TH-gels. Furthermore, the present studies showed that affinity purification and iodination did not influence the biologic characteristics of rabbit anti-TH antibodies in vivo,

since the serum half life of I^{125}-anti-TH did not differ from the half life of anti-TH determined by ELISA.

Since the whole kidney is not adequately freed of plasma proteins by perfusion with saline [14], a pair-label technique using normal rabbit IgG as a marker was required to determine the proportion of anti-TH that was nonspecifically trapped within tissue. Use of this technique allowed precise quantitation of the renal uptake of anti-TH and the demonstration that there was no specific uptake of anti-TH by other tissues. On the basis of these studies, we conclude that TH is present on cell surfaces only within the kidney. This conclusion is supported by two recent hybirdization studies indicating that the expression of TH messenger RNA is tissue-specific and, therefore, that TH is synthesized only in the kidney [33, 34]. It had been previously suggested that TH was also present in other tissues such as brain [35] and liver [36]. The basis for the results of these earlier localization studies is not known, but could relate to the detection of carbohydrate units that TH shares with other antigens, since TH is more than 25% carbohydrate [34].

The mechanism of immune deposit formation in the present model appears to involve the in situ combination of circulating anti-TH antibodies with TH, an antigen present on the surface of TAL cells [9, 10], a mechanism analogous to that involving other cell surface antigens [37, 38]. This interaction with antibody leads to antigenic modulation with an initial redistribution of antigen on the cell surface (patching) and subsequent decrease of antigen on the cell surface by endocytosis and/or shedding of the immune complexes formed. Subepithelial immune deposits in the distal nephron in rats and mice immunized with TH are associated with a reduction of basal cellular staining for TH [28, 30]. The capacity for antibody-induced antigenic modulation is an intrinsic property of individual antigens [39] and the rate of formation and fate of the immune complexes is greatly influenced by local conditions. For example, within the lung, interaction of antibodies to angiotensin converting enzyme with this antigen on the surface of endothelial cells in alvaeolar capillaries leads to a rapid depletion of antigen in this site; antibody deposits are absent by day 4, presumably on the basis of shedding of complexes into the circulation [40]. In contrast, within the ovary, immune complexes are detected later after injection of the same antibody. The interaction with antibody at the surface of oocytes produces antigenic modulation and prolonged retention of peripherally migrating immune deposits that have been shed into the polysaccharide milieu of the zona pellucida [41]. Recent in vitro studies using cultured glomerular epithelial cells have provided further evidence for the role of antibody-induced redistribution of Heymann antigen in the pathogenesis of this disease [42]. In passive Heymann nephritis, factors contributing to the slow build-up of subepithelial immune complexes within the glomerulus that continues over several days after injection of antibodies to the Heymann antigen appear to include the relatively small quantities of antigen normally present on the surface of glomerular epithelial cells, shedding and attachment of complexes to the GBM and de novo synthesis of this antigen [43].

The kinetics of renal anti-TH binding were markedly altered in proteinuric rats. The increased renal binding in these rats reflects primarily antibody bound to luminal tubular surfaces since immunofluorescence studies have shown that basal tubular deposits in proteinuric rats are decreased from those in the kidneys of normal rats that form exclusively basal deposits and bind a maximum of 20 μg of anti-TH after receiving 500 μg of anti-TH. Thus, more than 50 μg of the total of 70 μg bound in proteinuric rats (>70%) must be bound to the luminal surfaces of tubules. During the clearance phase, antibody was being selectively cleared into urine, since the ratio of anti-TH antibody clearance to rabbit IgG clearance into urine progressively increased to greater than unity by the end of the study period (Fig. 10). This appears to be the consequence of shedding of luminal immune complexes into urine. Our previous ultrastructural studies of proteinuric rats injected with anti-TH showed that very electron dense deposits were initially formed immediately adjacent to luminal TAL surfaces. These immune deposits were subsequently displaced from the cell surfaces by less electron dense fibrillar material with the fine structural features of TH [11]. The striking changes in TH-binding consequent to altered glomerular permeability suggest that this quantitative system may be useful in evaluating other experimental conditions.

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